International Journal of Medical Laboratory 2018;5(4):264-270.

International Journal of Medical Laboratory
Shahid Sadoughi University of Medical Sciences

Original Article

Evaluation of Ficolin-3 (FCN3) 1637delC (*rs28357092*) Frameshift Mutation in Iranian Type 2 Diabetic Subjects

Shaghayegh Pishkhan Dibazar[1] M.Sc., Ahmad Zavaran Hosseini[1*] Ph.D., Fatemeh Yari[2] Ph.D., Shirin Fateh[1] M.Sc., Mohammad Reza Deyhim[2] Ph.D.

[1]Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
[2]Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran.

Article history
Received 17 Mar 2018
Accepted 14 Nov 2018
Available online 27 Nov 2018

Key words
Diabetes mellitus
FCN3
Polymerase chain reaction
Polymorphism

A B S T R A C T

Background and Aims: Ficolins are proteins that bind to carbohydrates, act as opsonins and play an important role in innate immunity. Polymorphism in ficolin-3 gene (FCN3) can lead to complement deficiency and increase the risk of some disorders such as diabetes. The aim of this study was to investigate the frequency of *FCN3+1637delC* as a single nucleotide polymorphism in this gene in healthy and diabetic subjects of Iran.

Materials and Methods: Blood was taken from 36 diabetics and 37 healthy subjects who had referred to the Iranian Blood Transfusion Organization. Blood sugar was analyzed using a calorimetric method. After DNA extraction using salting out method, polymerase chain reaction (PCR) was carried out and the restriction fragment length polymorphism (RFLP) method was accomplished using ApaI restriction enzyme. Consequently, the resulted fragments were evaluated using electrophoresis on 2% L-agarose gel.

Results: Evaluation of the results indicated that the heterozygote form of single-nucleotide polymorphism *FCN3+1637delC* was seen in three samples (8.1%) of the studied healthy subjects and in two samples (5.6%) of the diabetic individuals. Besides, the homozygous form of the mutation was not seen in the studied healthy and diabetic subjects.

Conclusions: Results of this study showed that FCN3 variant of single-nucleotide polymorphism *FCN3+1637delC* was not considered as a risk factor for type 2 diabetes mellitus in Iranian subjects.

***Corresponding Author:** Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. Tel: +982182883090, Email: zavarana@modares.ac.ir

[DOI: 10.18502/ijml.v5i4.153]

Introduction

Ficolins are a set of innate immune molecules that bind to carbohydrate structures and acetylated compounds on the surface of microorganisms [1] and act like opsonins [2]. They also remove dead cells and activate complement system [3]. Complement is an important part of the innate immune system that eventually leads to pathogen lysis and cellular immune activation [4]. Defect in the complement cascade will increase risk of infectious diseases. Ficolins have been recognized in humans, rats, pigs and other animals. In human, they include FCN1 or M-ficolin, FCN2 or L- ficolin and FCN3 or H-ficolin [2]. FCN3 was primrily recognized as autoantigen in patients with systemic lupus erythematosus. It is a soluble pattern recognition molecule in the lectin complement pathway and the most abundant ficolin molecules in serum [5, 6]. FCN3 gene with 8 exon is located on chromosome 1 (1p36.11) and encodes 299 amino acids [7]. It is expressed primarily in the liver and lungs and at low levels in heart, kidneys, spleen, pancreas and placenta [8]. It binds to N-acetyl glucosamine, N-acetyl galactosamine and D-Focuse [9] and acts as an opsonin in lungs and blood to accelerate the phagocytosis[5] and is involved in the clearance of dead cells [10]. It also interacts with mannose binding lectin associated serine protease and activates lectin pathway of complement [11]. The absence or dysfunction of this molecule inhibits clearance of apoptotic cells so intracellular autoantigens will expose to the immune system by late apoptotic cells [12], therefore, FCN3 expression patterns and genetic variations (polymorphisms) are involved in the etiology of various diseases or predict them. There is a mutation (single-nucleotide polymorphism *FCN3+1637delC*, *rs28357092*) in exon 5 of FCN3 gene that leads to a shift in the reading frame and change in the c-terminal amino acid composition of the protein. The result is that the mutation causes a FCN3 with incomplete fibrinogen-like domain that fails to function properly so that FCN3 defect or deficiency will be observed. Homozygosity for this mutation is associated with severe recurrent infections and complement deficiency syndrome [10]. Complete lack of FCN3 is associated with impairment in removal of cellular residues and endogenous waste [13, 14], acute ischemic stroke [15], recurrent pulmonary infections, brain abscess [16] and type 2 diabetes mellitus (T2DM) [17].

T2DM is the most common metabolic diseases in the world [18]. Serum FCN3 varies significantly in individuals with normal glucose tolerance and those with T2DM [19] and low levels of FCN3 can be as a predictive factor in diabetes [17, 20]. The aim of this study was to evaluate the *FCN3+1637delC* frameshift mutation in Iranian T2DM subjects.

Materials and Methods

Subjects

Thirty six diabetic subjects were chosen according to the American Diabetes Society criteria. The control group was selected from

[DOI: 10.18502/ijml.v5i4.153]

37 healthy volunteers who had referred to the Iranian Blood Transfusion Organization based on their normal glucose indices. The mean age of the patients and control group subjects was 56±16 and 41±13 years, respectively. The protocol was approved by the Ethical Committee of Tarbiat Modares University, Tehran, Iran.

Blood sugar measurement

Blood sugar was measured by a diagnostic kit (Darvash, Iran) and Hitachi 911 auto analyzer (Roche, Germany).

DNA extraction

DNA was extracted from the whole blood samples using salting-out method as below: 1.5 ml of red blood cells lysis buffer; 0.32 M Sucrose (Merck, Germany), 10 mM Tris-HCl (Merck, Germany), 5 mM MgCl2 (Merck, Germany), and 1% Triton X-100 (Sigma, Japan). The pH was adjusted on 7.5. The buffer was added to the blood samples, then centrifuged at 1402 × g for 10 min and rinsed. This process was repeated three times. After removing the supernatant, 800 µl of white blood cells lysis buffer was used; 10 mM Tris-HCl, 400 mM NaCl, 2 mM Na2EDTA and the pH were adjusted on 8.2. Subsequently, 100 µl of 6 M NaCl and 600 µl of chloroform were added. Then centrifugation was carried out at 8765×g for 5 min. After collecting the chloroform supernatant, 800 µl of isopropanol (-20°C) was added. In this step, DNA coils was observed and rinsed with 100 µl 70% ethanol at 12000×g for 5 min. Ultimately DNA containing microtube was placed in a heat block device (Techne, UK) with 60°C heat. Later 100 µl of distilled water was added and DNA was dissolved. Absorbance was read with Nano Drop spectrophotometer (WPA, UK) at three wavelengths of 230, 260 and 280 nm. The ratio of 260/280 nm was considered for purity estimation of DNA.

Polymerase chain reaction (PCR)

PCR was carried out using the primers (exon 5; forward:5′-GATCACATGGTGTTGGGTGT-3′, reverse: 5′-TTCACTCTTTTCACCCAGGC-3′). For each PCR reaction, 100 ng of DNA was added to the reaction mixture containing a final concentration of 0.4 µM of each primer, 0.2 mM of dNTPs, 4 mM of MgCL2, 1x PCR buffer (pH 8.5), and 5µl/unit of Taq DNA polymerase. The reaction was carried out in a thermal cycler (Rotor-Gene Q, Qiagen) for 30 cycles, with each cycle consisting of 50 s at 94°C, 32 s at 69°C and 40 s at 71°C. The first cycle was preceded by a denaturation step at 95°C for 4 min, and the last one was followed by an extension step at 72°C for 6 min. Electrophoresis of PCR products was performed with 2% agarose gel in 150 V for 30 min. Gel was stained with ethidium bromide. Amplified fragments were observed under ultraviolet light with a wavelength of 320 nm.

Polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP)

PCR was followed by PCR-RFLP method. For digestion of the PCR product, 4 µl of PCR product was digested with 10U of the restriction enzyme; ApaI (Fermentase, Germany) according to the kit protocol. Additionally, few PCR product samples of FCN3 gene (exon 5) were sequenced (SinaClon Co., Iran) to resolve the ambiguities.

Statistical Analysis

Statistical analysis was performed by means of the SPSS v.16.0 (SPSS Inc., Chicago, IL, USA).

[DOI: 10.18502/ijml.v5i4.153]

Hardy-Weinberg analysis was applied to compare the observed and expected genotype frequencies by chi-square test. Moreover, odds ratios and 95% confidence interval (CI) were estimated for both the patients and healthy controls.

Results

The characteristics of the samples

The characteristics of the patients and control subjects are shown in Table 1. Blood sugar levels were 212.4±58 (mg/dl) in the patients and 86.1±8 (mg/dl) in the control group.

PCR-RFLP results

The extracted DNA samples had high purity (260/280 nm=1.8) and concentration (~300 µg/ml). FCN3 PCR product size was 650 bp in electrophoresis (Fig. 1). After digestion of the PCR product with ApaI restriction enzyme, the resulting fragments were 266 bp and 384 bp in wild type/ wild type genotype (without polymorphism). In the case of heterozygosity (wild type/deletion genotype), three bands were detected; 266, 384 and 650 bp (Fig. 2). None of the specimens were homozygote for the mutation (with only the 650 bp). The sequence based typing showed 99% identity of PCR product with human FCN3 gene.

FCN3+1637delC deletion mutation in the studied subjects

The allele frequencies and genotype distributions for *FCN3+1637delC* mutation were determined in the case and control subjects (Table 2). According to the data 94.4% of diabetic and 91.9% of normal samples did not show *1637delC* allele of ficolin gene. Totally, 5.6% and 8.1% of the studied samples indicated heterozygosity for this allele in the diabetic and normal samples, respectively (95% CI=0.236-9.552, p>0.05). Moreover, no significant correlation was identified between the polymorphism of FCN3 and the risk of T2DM in Iranian subjects.

Table 1. The age and sex status and FBS levels in the studied subjects of this study

	Patients (N=36)	Controls (N=37)
Female: male	19:17	16:21
Age	56±16	41±13
Fasting blood sugar	212.4±58	86.1±8

Data presented as mean±SD

Table 2. single-nucleotide polymorphism FCN3 +1637delC genotype and allele frequencies; distribution in Iranian patient and control subjects

Genotype/Allele	Patients N=36 (%)	Controls N=37 (%)	OR	95%CI
FCN3+1637delC				
WW	34 (94.4%)	34 (91.9%)	1.500	0.236-9.552
WD	2 (5.6%)	3 (8.1%)	1.500	0.236-9.552
DD	0 (0%)	0 (0%)	-	-
W	70 (97.2%)	71 (95.9%)	1.479	0.240-9.122
D	2 (2.8%)	3 (4.1%)	1.479	0.240-9.122

OR= Odds ratio; CI= Confidence interval; WW= Wild type/wild type; WD= Wild type/deletion; DD= Deletion/deletion. There was no statistically significant difference in the distributions of *FCN3+1637delC* polymorphisms between diabetic patients and control subjects.

[DOI: 10.18502/ijml.v5i4.153]

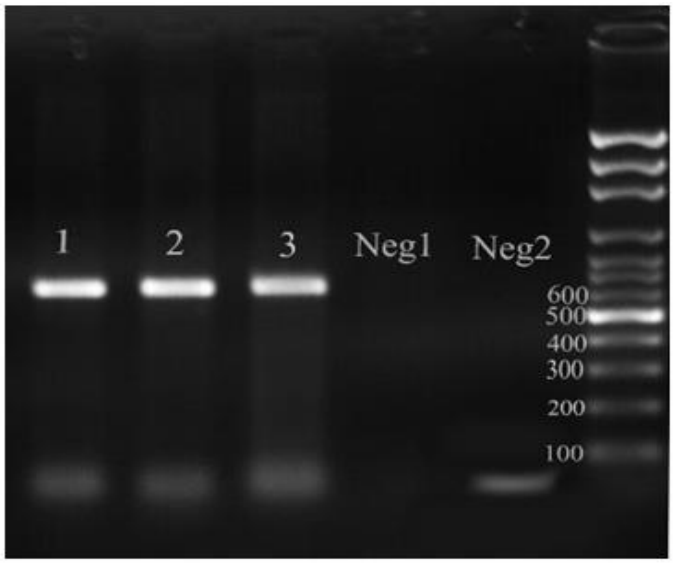

Fig. 1. The PCR product of exon 5 of FCN3 gene. Lanes 1-3; FCN3 PCR product (650 bp), Neg1; Negative control without primer, Neg2; negative control without DNA, 100-bp DNA ladder.

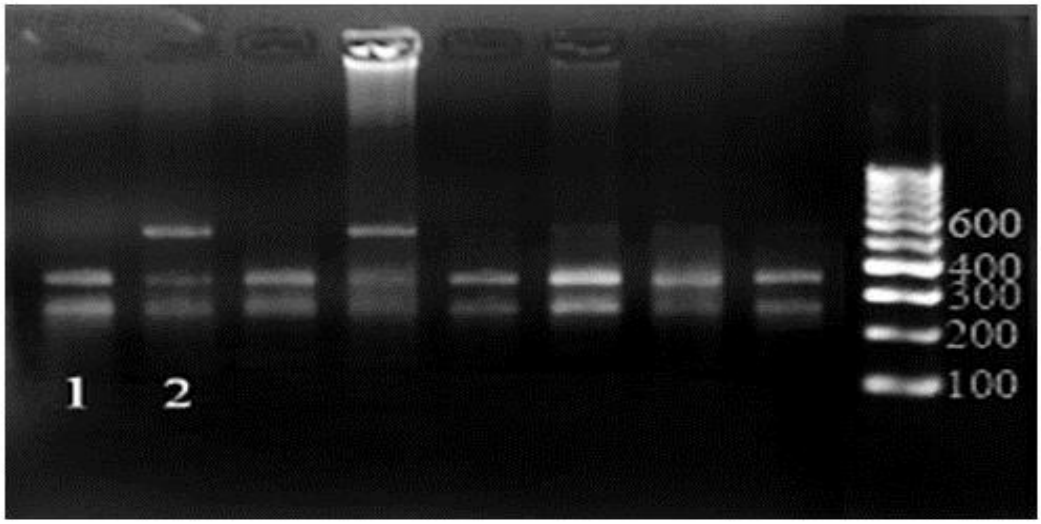

Fig. 2. Polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis of FCN3 gene (exon 5) by ApaI restriction enzyme in the studied subjects. Lane 1; Two bands of 266 bp and 384 bp represent wild type/ wild type genotype (without polymorphism), while in the case of heterozygosity (wild type/ deletion genotype), three bands of 266, 384 and 650 bp can be detected in lane 2.

Discussion

In this study, we observed that 5.6% of the diabetic and 8.1% of normal subjects are heterozygous for FCN3 frameshift mutation (*rs28357092*); the frequency of wild type homozygote was 94.4% in diabetic and 91.9% in normal subjects. The results showed higher frequency for wild type/deletion genotype in Iranian normal subjects compared with other studies. For example in (237) healthy Danish Caucasians, the frequencies of wild type/deletion heterozygote and wild type homozygote were 2.1 and 97.9 percent [7] and about 1.8% of the 1282 immunodeficient patients in Munthe-Fog and colleagues study

[DOI: 10.18502/ijml.v5i4.153]

were *FCN3+1637delC* mutation, giving rise to an allele frequency of 0.01, which is the same as that observed in healthy white population [21]. The difference between our results and Munthe-Fog may be related to the number of the studied population or the method of the study. In another study, *FCN3+1637delc* polymorphism was assessed in 190 leprosy patients in contrast to 245 control in Brazil. Allele frequency was 2.4% and 2.2% [22]. Prevalence of heterozygosity for the *1637delC* allele in 120 mannan-binding lectin deficient Icelandic patient was 4.2% [23].

Conclusion

Our study showed that FCN3 frameshift mutation do not associate with blood sugar levels. Also there was no relation between this polymorphism and the gender. The frequency of FCN3 frameshift mutation (rs28357092) or *FCN3+1637delC* was not statistically significant between the patient and control groups. So there was no association between single nucleotide polymorphism *FCN3+1637delC* and the risk of T2DM in the Iranian subjects. However, with regards to the FCN3 role in the immune system, further studies should be conducted with larger sample sizes.

Conflict of interest

None of the authors have any conflicts of interest to declare.

Acknowledgements

This work has been performed with a grant from Tarbiat Modares University. The authors wish to thank the Iranian Blood Transfusion Organization (Tehran, Iran) for the technical support.

References

[1]. Hummelshoj T, Fog LM, Madsen HO, Sim RB, Garred P. Comparative study of the human ficolins reveals unique features of Ficolin-3 (Hakata antigen). Mol Immunol. 2008; 45(6): 1623-632.

[2]. Hummelshøj T, Ma YJ, Munthe-Fog L, Bjarnsholt T, Moser C, Skjoedt MO, et al. The Interaction Pattern of Murine Serum Ficolin-A with Microorganisms. PLoS One 2012; 7(5): e38196.

[3]. Thielens N, Gaboriaud C, Arlaud G. Ficolins: innate immune recognition proteins for danger sensing. Inmunología 2007; 26(3): 145-56.

[4]. Runza VL, Schwaeble W, Männel DN. Ficolins: novel pattern recognition molecules of the innate immune response. Immunobiology 2008; 213(3-4): 297-306.

[5]. Matsushita M, Fujita T. The role of ficolins in innate immunity. Immunobiol. 2002; 205(4-5): 490-97.

[6]. Zacho RM, Jensen L, Terp R, Jensenius JC, Thiel S. Studies of the pattern recognition molecule H-ficolin specificity and purification. J Biol Chem. 2012; 287(11): 8071-81.

[7]. Munthe-Fog L, Hummelshøj T, Ma YJ, Hansen BE, Koch C, Madsen HO, et al. Characterization of a polymorphism in the coding sequence of FCN3 resulting in a Ficolin-3 (Hakata antigen) deficiency state. Mol Immunol. 2008; 45(9): 2660-666.

[8]. Garred P, Honoré C, Ma YJ, Rørvig S, Cowland J, Borregaard N, et al. The genetics of ficolins. J Innate Immun. 2010; 2(1): 3-16.

[9]. Ma YJ, Doni A, Hummelshøj T, Honoré C, Bastone A, Mantovani A, et al. Synergy between ficolin-2 and pentraxin 3 boosts innate immune recognition and complement deposition. J Biol Chem. 2009; 284(41): 28263-8275.

[10]. Hein E, Honoré C, Skjoedt MO, Munthe-Fog L, Hummelshøj T, Garred P. Functional analysis of Ficolin-3 mediated complement activation. PLoS One 2010; 5(11): e15443.

[11]. Hummelshoj T, Munthe-Fog L, Madsen HO, Fujita T, Matsushita M, Garred P. Polymorphisms in the FCN2 gene determine serum variation and function of Ficolin-2. Hum Mol Genet. 2005; 14(12): 1651-658.

[12]. Honoré C, Hummelshoj T, Hansen BE, Madsen HO, Eggleton P, Garred P. The innate

[DOI: 10.18502/ijml.v5i4.153]

immune component ficolin 3 (Hakata antigen) mediates the clearance of late apoptotic cells. Arthritis Rheum. 2007; 56(5): 1598-607.

[13]. Michalski M, Szala A, Swierzko AS, Lukasiewicz J, Maciejewska A, Kilpatrick DC, et al. H-ficolin (ficolin-3) concentrations and FCN3 gene polymorphism in neonates. Immunobiol. 2012; 217(7): 730-37.

[14]. Endo Y, Matsushita M, Fujita T. Role of ficolin in innate immunity and its molecular basis. Immunobiol. 2007; 212(4-5): 371-79.

[15]. Füst G, Munthe-Fog L, Illes Z, Széplaki G, Molnar T, Pusch G, et al. Low ficolin-3 levels in early follow-up serum samples are associated with the severity and unfavorable outcome of acute ischemic stroke. J Neuroinflamm. 2011; 8: 185.

[16]. Honoré C, Rørvig S, Hummelshøj T, Skjoedt MO, Borregaard N, Garred P. Tethering of Ficolin-1 to cell surfaces through recognition of sialic acid by the fibrinogen-like domain. J Leukoc Biol. 2010; 88(1): 145-58.

[17]. Chen H, Lu J, Chen X, Yu H, Zhang L, Bao Y, et al. Low serum levels of the innate immune component ficolin-3 is associated with insulin resistance and predicts the development of type 2 diabetes. J Mol Cell Biol. 2012;4(4):256-7.

[18]. Korc M. Diabetes mellitus in the era of proteomics. Mol Cell Proteomics 2003; 2(6): 399-404.

[19]. Li RX, Chen HB, Tu K, Zhao SL, Zhou H, Li SJ, et al. Localized-statistical quantification of human serum proteome associated with type 2 diabetes. PLoS One 2008; 3(9): e3224.

[20]. Tso AW, Xu A, Sham PC, Wat NM, Wang Y, Fong CH, et al. Serum adipocyte fatty acid–binding protein as a new biomarker predicting the development of type 2 diabetes: A 10-year prospective study in a Chinese cohort. Diabetes Care. 2007; 30(10): 2667-672.

[21]. Munthe-Fog L, Hummelshøj T, Honoré C, Madsen HO, Permin H, Garred P. Immunodeficiency associated with FCN3 mutation and ficolin-3 deficiency. N Engl J Med. 2009; 360(25): 2637-644.

[22]. Andrade FA, Beltrame MH, Bini VB, Gonçalves LB, Boldt ABW, de Taborda Messias- Reason IJ. Association of a new FCN3 haplotype with high ficolin-3 levels in leprosy. PLoS Negl Trop Dis. 2017; 11(2): e0005409.

[23]. Bjarnadottir H, Thorsteinsdottir V, Jorgensen GH, Arnardottir M, Ludviksson BR. Mannan-binding lectin (MBL) deficient individuals with the O/O genotype are highly susceptible to gastrointestinal diseases. J Clin Cell Immunol. 2014; 5(1): 182.

[DOI: 10.18502/ijml.v5i4.153]